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Brain Research 964 (2003) 159–163
BRAIN RESEARCH
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Research report

Cortico-striatal synaptic plasticity in endothelial nitric oxide synthase deficient mice

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Accepted 26 November 2002

Abstract

Nitric oxide (NO) is a retrograde messenger involved in the processes of learning and memory. The role of the endothelial isoform of nitric oxide synthase (eNOS) in striatal synaptic plasticity was investigated in eNOS-deficient ($eNOS^{-/-}$) and wild type (WT) mice. Tetanic stimulation of cortical afferents in WT mice evoked either long-term potentiation (LTP), or long-term depression (LTD) of cortico-striatal transmission. Both these plasticity related phenomena were NMDA-receptor-dependent; LTD was blocked by sulpiride, a dopamine D2-receptor antagonist. LTP occurrence in slices from $eNOS^{-/-}$ mice was significantly reduced when compared with WT mice. The NOS inhibitor NL-ARG reduced the occurrence of LTP and increased the occurrence of LTD in WT mice, resembling the balance of LTP/LTD in $eNOS^{-/-}$ mice. Impairment of NO-synthesis thus shifts striatal plasticity towards LTD. This indicates a possible involvement of eNOS from endothelia in neuronal modulation.

Theme: Excitable membranes and synaptic transmission

Topic: Long-term potentiation: pharmacology; Long-term potentiation: physiology

Keywords: Endothelial nitric oxide synthase; Long-term potentiation; Long-term depression; NMDA-receptor

1. Introduction

The long-term storage of information within neural circuits involves activity-dependent changes in the efficacy of synaptic transmission [1]. The striatum is a brain region involved in the regulation of movements, a task depending on memory processing and storage [8]. GABAergic medium spiny neurons, which represent the large majority of the neuronal population of the striatum, project to the substantia nigra and the globus pallidus. Dopaminergic fibers from the substantia nigra pars compacta modulate cortico-striatal glutamatergic transmission. Two different forms of plasticity have been found at cortico-striatal synapses: long-term depression (LTD) and long-term potentiation (LTP) [2,12], presumable correlates of motor learning. NO is involved in synaptic plasticity in the striatum [4] and elsewhere, e.g. [16]. The neuronal but so far not the endothelial NOS isoform was detected in some striatal interneurons with a large axonal arborisation [11]. On the other hand, in the hippocampus, nNOS is localized to GABAergic interneurons whereas eNOS is found in pyramidal neurons [6,5,13]. A microdialysis study in eNOS- and nNOS-deficient mice revealed differences in NMDA-stimulated amino acid release in the striatal probes: GABA release was reduced in $eNOS^{-/-}$ (not $nNOS^{-/-}$) mice, glutamate release showed an opposite pattern (reduction in $nNOS^{-/-}$ but not $eNOS^{-/-}$ -mice) [10]. The role of eNOS in striatal synaptic plasticity has not been studied so far. NOS-positive neurons may control local blood flow in the striatum by releasing NO, and NO from endothelia may in principle also affect neighboring neurons. In rat striatal slices the NO/cGMP/PKG cascade was shown to be required for the induction of LTD in the

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0006-8993/02/$ – see front matter
doi:10.1016/S0006-8993(02)04121-5

medium spiny projecting neurons [4]. We have previously found a defect in hippocampal and neocortical LTP in eNOS$^{-/-}$ mice [9,15] and present now an investigation on striatal synaptic plasticity in these rodents.

2. Materials and methods

Six to 8-week-old wild type (C57/Bl6; $n=19$) and eNOS $^{-/-}$ ($n=19$) animals of either sex were killed by swift decapitation. Their brains were removed rapidly from the skull and immersed in ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM): 120 NaCl, 1.8 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 2.0 $CaCl_2$, 25 $NaHCO_3$ and 10 glucose bubbled with a 95% O_2–5% CO_2 mixture at pH 7.4. Using a vibratome, 400–500 μm slices were cut at approximately 20° to the horizontal plane. Each slice contained, after dissection from surrounding tissue, the anterodorsal part of the striatum, white matter, and a portion of anterior cortex. After 1–2 h of preincubation at room temperature, slices were transferred to a submersion-type recording chamber where they were continuously perfused with ACSF at a flow rate of 1.5–2 ml/min at 32 °C.

Field potentials were evoked by electrical stimulation of cortico-striatal fibers (0.05 Hz, 80 μs duration) using a 50 μm bipolar nickel–chromium electrode located at white matter between cortex and striatum. Glass micropipettes filled with ACSF (3–4 MΩ) were placed in the striatum at a distance of 200–300 μm for recording field potentials. pCLAMP6 (Axon Instruments) software was used to analyze the data off-line. After the response had stabilized in each slice, test stimulus intensity (60% of maximal response) was selected and 25 min baseline was recorded (15 responses were averaged for each data point). A high frequency tetanus (three trains of 10 pulses at 100 Hz, with 20 s inter-train intervals) was delivered at unchanged intensity. In slices to be tested in presence of N^G-Nitro-L-Arginine methyl ester (NL-ARG, 200 μM), a stable baseline was first obtained, then slices were perfused with this drug for at least 60 min before the tetanic stimulation and throughout the experiment. All values were normalized to the mean value over the 30-min control period. The data were expressed as mean±S.E.M. and analyzed statistically using Student's t-test and Fisher's exact probability test; P values equal or less than 0.05 were considered significant. Drugs were obtained from Sigma, Deisenhofen, Germany.

3. Results

The characteristic field potential evoked by cortical white matter stimulation (Fig. 1A) consisted of two negative spikes, the first (N1) reflecting a fiber potential and direct activation of medium spiny neurons, the second (N2) being a synaptically induced wave (Fig. 1B). High frequency stimulation produced a long-term potentiation (LTP, >110% of control amplitude), an enduring decrease (long-term depression, LTD, <90% of control) or no long-term change (between 90–110% of control) in the synaptically evoked field potentials averaged during the time period between 60 and 90 min after tetanization. Cortico-striatal slices ($n=105$) from 38 animals were tested.

Following tetanic stimulation LTP was observed in the majority of striatal slices from WT mice (58%), LTD was seen in 37% (Table 1 and Fig. 1C). Bath application of the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV, 50 μM) 20 min before and during tetanization period prevented the development of LTP and LTD (Fig. 1D). No changes of the N2 field potentials were recorded in 8 slices: 90 min after tetanization the amplitude was 99.4±2.1% of baseline. One slice displayed LTD (88.3% of baseline). The difference between LTD occurrence in control and in the presence of APV was highly significant ($P<0.001$, Fisher's exact probability test).

In the presence of the antagonist at dopamine D2-receptors, sulpiride (1 μM, applied during the whole registration period), LTD was never seen; LTP occurrence and magnitude were not significantly different from control (50% and 127.3±9.5%, $n=4$, respectively). In 4 slices no long-lasting changes occurred in the field potential amplitude after tetanization (103±2.5% over baseline).

WT and eNOS$^{-/-}$ mice did not show a significant difference in LTP and LTD magnitudes (Table 1), but the occurrence of LTP (versus LTD and no changes) was significantly lower ($P<0.05$, Fisher's exact probability test).

Next, we have tested LTP/LTD occurrence in WT and KO animals in magnesium-free solution. The idea behind these experiments was to facilitate LTP (versus LTD) induction [4,12]. The removal of Mg^{2+}-ions from the medium led to an increase in the amplitudes of LTP both in control and knockout animals (Table 1). However, the differences in LTP magnitudes between control and magnesium-free medium were not significant (Student's t-test, $P=0.11$ WT and $P=0.25$ KO). LTP occurrence was significantly reduced in KO mice in comparison with WT ($P<0.01$, Fisher's exact probability test, Fig. 2). The number of slices displaying LTD or no long-term changes was correspondingly increased (Fig. 2C).

Treating of the slices from WT mice in Mg^{2+}-free solution with the NOS inhibitor NL-ARG decreased the occurrence of LTP (from 75% to 40%, $P=0.05$, Fisher's exact probability test) and increased both, the occurrence of LTD (from 17% to 30%) and the number of slices with unchanged synaptically evoked potentials (from 8% to 30%, Fig. 2C). Thus, NL-ARG-exposure shifted the LTP/LTD occurrence balance in the same direction that was seen in eNOS-deficient mice. The magnitude and time

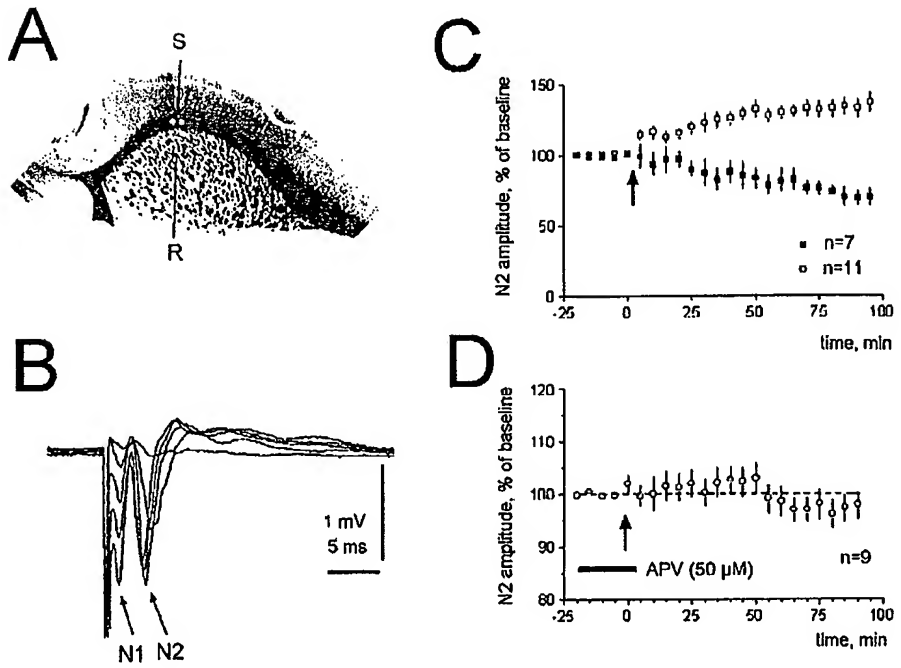

Fig. 1. Tetanic stimulation of cortical afferents evokes LTD or LTP in the majority of striatal slices from mice. A: Stimulating (S) and recording (R) electrode positions are indicated with arrows on the photograph of a cortico-striatal slice. B: Example of striatal field potentials evoked by different stimulus strength. The N1 component reflects a fiber potential and direct activation of medium spiny neurons; N2 is a synaptically induced wave. Each trace is an average of 15 individual field potentials. C: The average diagrams show the time-course of changes in the second negative component of the field potential (N2) after tetanization (arrow). All cases of LTP (open square) or LTD (closed square) occurrence were averaged. D: Absence of long-term changes of field potentials after tetanization in the presence of APV.

course of LTP and LTD in slices from WT animals were similar with or without NL-ARG (Fig. 2D, Table1).

4. Discussion

We describe here changes in cortico-striatal synaptic plasticity in mice with a targeted mutation of the eNOS-gene: the occurrence of LTP is decreased while that of LTD is increased in eNOS$^{-/-}$-mice. Both LTP and LTD could be evoked by the same high frequency stimulation in normal recording solution. The same pattern was found by some previous investigators [12,7] but not by others [2] in rat slices. Methodological or species differences may be responsible for this discrepancy. A mouse slice of the same thickness contains more of the striatal circuitry and the plane of cutting is obviously of importance here.

We observed a complete APV-dependence of LTP, indicating the critical role of NMDA receptors in wild-type mice. Our failure to record LTD in APV-treated slices of wild type mice is not in keeping with the data from rats where LTD was found to be NMDA-independent [3], but it is in agreement with Spencer and Murphy [12], who regularly obtained LTP in striatal saggital slices in response to white matter stimulation in normal (magnesium-containing) solution using experimental protocols similar to ours. These authors found that LTD was not the predominant form of synaptic plasticity induced by affer-

Table 1
LTP and LTD in the different mouse groups

Genotype, n slices, total	LTP		LTD	
	magnitude, %	n slices	magnitude, %	n slices
WT, n=19	133.2±6.3	11	68.3±4.9	7
KO, n=19	141.6±8.1	5	77.2±5.5	9
WT, Mg^{2+}-free solution, n=24	169.6±17.0	18	80.75±2.3	4
KO, Mg^{2+}-free solution, n=24	181.9±23.9	9	64.4±5.1	8
WT, Mg^{2+}-free + NL-ARG, n=10	145.5±17.2	4	73±7.2	3

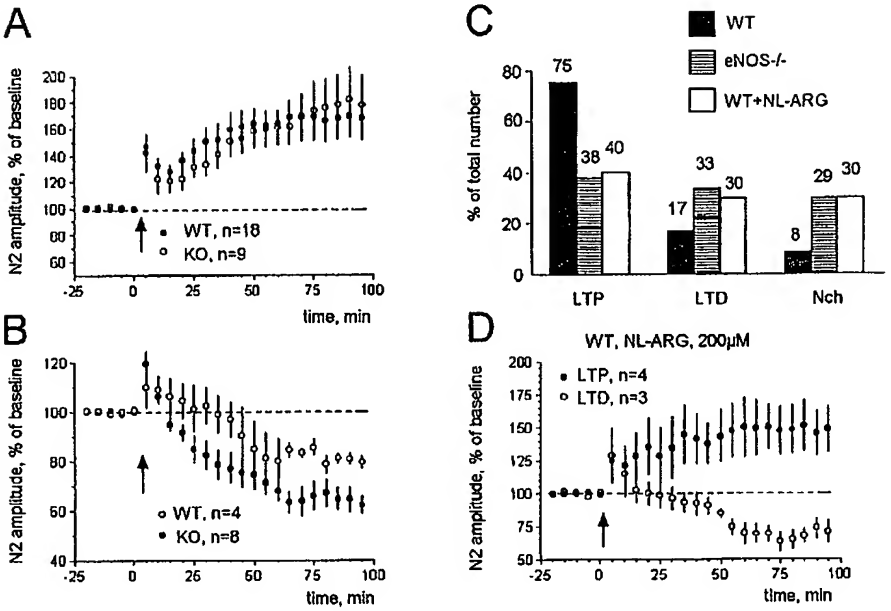

Fig. 2. Wild type (WT) and eNOS-deficient (KO) mice display no difference in either LTP (A) or LTD (B) magnitudes, but KO mice show a reduced LTP-occurrence (C). D: Time-course of N2 changes (LTP, open circles and LTD, closed circles) after tetanization (indicated by arrow) in the presence of N^G-Nitro-L-Arginine methyl ester (NL-Arg) in wild type animals. All experiments in magnesium-free solution.

ent tetanic stimulation, dependent on NMDA- and dopamine-receptor (D1 and D2) activation. In agreement with this study we observed LTD in 37% of wild type animals in normal and 17% in magnesium-free medium. The change in LTP/LTD balance observed in eNOS-deficient mice was due to the lack of NO production as an acute block of NO synthesis mimicked this effect.

The shift in LTP/LTD balance toward the latter in eNOS-deficient mice is in contrast to the finding by Calabresi et al. [4] that the NO/cGMP/PKG pathway is needed for the expression of LTD in rat striatal slices. The phenomena described in our study in mice are also different from this group's result in the rat as we observed LTP and LTD and both were NMDA-dependent. We see two possible reasons for that: (i) our recordings were done in the frontal striatum (see Fig. 1A), where LTP is the predominant form of plasticity [12], (ii) during tetanic stimulation we did not increase the intensity of the stimulus as Calabresi et al. did [4]. We have shown previously that tetanization strength is critical for the involvement of NO in hippocampal LTP [15,16]: only a weak tetanization paradigm leads to the NO-dependent LTP. Thus, complexity of striatal circuitry or/and tetanization paradigms may be responsible for the modulation of synaptic plasticity in different directions by NO, obtained by different investigators.

Modeling of perivascular O_2 and NO concentration gradients indicates that targets 200 µm from the vessel wall may still be reached by sufficient concentrations of NO [14]. In the light of the probable absence of eNOS in striatal neurons our results indicate the intriguing possibility that NO released from non-neuronal sources like the endothelia participates in the modulation of cortico-striatal plasticity.

Acknowledgements

Supported by Deutsche Forschungsgemeinschaft, SFB 194, B13; and SPP 1026 to H.L.H. and a Lise-Meitner-Stipendium to O.A.S.

References

[1] T.V. Bliss, G.L. Collingridge, A synaptic model of memory: long-term potentiation in the hippocampus, Nature 361 (1993) 31–39.
[2] P. Calabresi, A. Pisani, N.B. Mercuri, G. Bernardi, The corticostriatal projection: from synaptic plasticity to dysfunctions of the basal ganglia, Trends Neurosci. 19 (1996) 19–24.
[3] P. Calabresi, D. Centonze, P. Gubellini, G.A. Marfia, A. Pisani, G. Sancesario, G. Bernardi, Synaptic transmission in the striatum: from plasticity to neurodegeneration, Prog. Neurobiol. 61 (2000) 231–265.
[4] P. Calabresi, P. Gubellini, D. Centonze, G. Sancesario, M. Morello, M. Giorgi, A. Pisani, G. Bernardi, A critical role of the nitric oxide/cGMP pathway in corticostriatal long-term depression, J. Neurosci. 19 (1999) 2489–2499.
[5] R.J. Cork, M.L. Perrone, D. Bridges, J. Wandell, C.A. Scheiner, R.R. Mize, A web-accessible digital atlas of the distribution of nitric oxide synthase in the mouse brain, Prog. Brain Res. 118 (1998) 37–50.

[6] J.L. Dinerman, T.M. Dawson, M.J. Schell, A. Snowman, S.H. Snyder, Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 4214–4218.
[7] S.V. Dos, J.P. Walsh, Modulation of long-term synaptic plasticity at excitatory striatal synapses, Neuroscience 90 (1999) 1031–1041.
[8] A.M. Graybiel, The basal ganglia, Trends Neurosci. 18 (1995) 60–62.
[9] S. Haul, A. Godecke, J. Schrader, H.L. Haas, H.J. Luhmann, Impairment of neocortical long-term potentiation in mice deficient of endothelial nitric oxide synthase, J. Neurophysiol. 81 (1999) 494–497.
[10] T. Kano, S.M. Shimizu, P.L. Huang, M.A. Moskowitz, E.H. Lo, Effects of nitric oxide synthase gene knockout on neurotransmitter release in vivo, Neuroscience 86 (1998) 695–699.
[11] Y. Kawaguchi, Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum, J. Neurosci. 13 (1993) 4908–4923.
[12] J.P. Spencer, K.P. Murphy, Bi-directional changes in synaptic plasticity induced at corticostriatal synapses in vitro. [In Process Citation], Exp. Brain Res. 135 (2000) 497–503.
[13] A.M. Teichert, T.L. Miller, S.C. Tai, Y. Wang, X. Bei, G.B. Robb, M.J. Phillips, P.A. Marsden, In vivo expression profile of an endothelial nitric oxide synthase promoter–reporter transgene, Am. J. Physiol. Heart Circ. Physiol. 278 (2000) H1352–H1361.
[14] D.D. Thomas, X. Liu, S.P. Kantrow, J.R. Lancaster Jr., The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O_2, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 355–360.
[15] R.I. Wilson, J. Yanovsky, A. Godecke, D.R. Stevens, J. Schrader, H.L. Haas, Endothelial nitric oxide synthase and LTP [letter], Nature 386 (1997) 338.
[16] R.I. Wilson, A. Godecke, R.E. Brown, J. Schrader, H.L. Haas, Mice deficient in endothelial nitric oxide synthase exhibit a selective deficit in hippocampal long-term potentiation, Neuroscience 90 (1999) 1157–1165.